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(71) Applicant *(for all designated States except US)*: REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 300 Lakeside Drive, 22nd Floor, Oakland, CA 94612-3550 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : ABRAHAM, Edward [US/US]; 9001 Ashcroft Avenue, Los Angeles, CA 90048 (US). CHANG, Yi-Han [US/US]; 1024 Somera Road, Los Angeles, CA 90077 (US).

(74) Agents: WETHERELL, John, R., Jr. et al.; Spensley Horn Jubas & Lubitz, 1880 Century Park East, Suite 500, Los Angeles, CA 90067 (US).

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(54) Title: LIPOSOMAL POLYSACCHARIDE VACCINES

(57) Abstract

Methods and compositions for inducing a mucosal immune response to liposomally encapsulated polysaccharide antigen.

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LIPOSOMAL POLYSACCHARIDE VACCINES

This application is a continuation-in-part of U.S. Serial No. 07/699,144 filed May 13, 1991.

1. Field of The Invention

The present invention relates to methods and compositions for inducing a mucosal immune response to polysaccharide antigen encapsulated in a liposome.

2. Related Art

Despite the availability of intensive care, increasingly powerful antimicrobial agents, and rigorous prophylactic therapy, infections leading to multiple organ system failure (MOSF) remain the major cause of late morbidity and mortality after such immunosuppressive events as trauma, hemorrhage, and burns. It has been estimated that from 60-88% of deaths occurring more than 7 days after hemorrhage, blunt trauma, or thermal injury are caused by sepsis. Nosocomial pneumonia is frequent after injury, and often contributes to multiple organ system failure, morbidity, and mortality in this setting. As many as 30-70% of all severely injured patients develop respiratory tract infections in the intensive care unit.

Secretory immunoglobulin A is the predominant immunoglobulin at mucosal surfaces, such as the lung or intestine, and microbial antigen-specific sIgA generated in gastro-intestinal or respiratory mucosa confers protection against pathogens infecting the host at or via these surfaces. Protection against mucosally associated infection correlates best with the number of antigen-specific plasma cells and secretory antibody levels in mucosal sites, and not with serum IgG antibody levels or splenic plasma cell responses.

In contrast, sIgA plays a minor role in protecting the host in systemically invasive infections, such as bacteremia.

T and B cells found in mucosal sites such as the lung, Peyer's patches, and intestinal lamina propria constitute a common mucosal immune system, and a critical first line of defense against bacterial infection. The mucosal B cells, unlike systemic B cells, primarily produce sIgA, and antigen-specific antibody responses in mucosal sites often do not parallel those occurring in systemic lymphoid organs, such as the spleen and lymph nodes. sIgA responses that originate in one mucosal site, such as the gut associated lymphoid tissue (GALT), disseminate to other mucosally associated lymphoid sites, such as the lung, tonsils, and salivary glands. This migration of sIgA secreting plasma cells between mucosal sites forms the basis for oral and intranasal immunization strategies aimed at improving pulmonary immunity to respiratory pathogens. In particular, this strategy has been used (Mestecky, *J.Clin.Immunol.*, 7:265, 1987; Liang, *et al.*, *J.Immunol.*, 143:484, 1989; and Tamara, *et al.*, *Vaccine*, 7:314, 1989) in developing vaccines against protein antigens associated with viral infections, such as influenza, where increased resistance to infections originating at pulmonary surfaces has been shown to accompany enhanced viral antigen specific pulmonary secretary antibody production and B cell response.

Enhancement of the pulmonary mucosal immune response to protein antigens, to more than 100 times baseline, has been achieved through oral or intranasal administration of the antigen of interest and an adjuvant, such as cholera toxin (Elson, *et al.*, *J.Immunol.*, 132:2736, 1984), glutaraldehyde deactivated cholera toxin (Liang, *et al.*, *J.Immunol.*, 141:1495, 1988), B-subunit of cholera toxin (McKenzie, *et al.*, *J.Immunol.*, 133:1818, 1984) and antigen encapsulated in liposomal formulations. However, neither oral nor intranasal immunization with combinations of bacterial polysaccharide antigens and adjuvants has been successfully reported to date.

The mechanism of action of cholera toxin derived products as adjuvants appears to center about the affinity of the cholera toxin B subunit for GMI gangliosides on cell surfaces. This property of the cholera toxin B subunit enhances delivery of associated antigens to the surfaces of mucosally associated cells initially involved in a localized and then, subsequently, in a generalized mucosal IgA response to the presented antigen. Deactivated cholera toxin and the B subunit of cholera toxin have been proposed to be appropriate for human oral vaccines since they lack significant toxicity. In particular, the cholera toxin B subunit has been used successfully as a component of human and animal vaccines against protein antigens of influenza and cholera. An advantage of liposomes is their nontoxic nature. Further, liposomes have been shown in several systems to have potent immunoenhancing properties on the mucosal immune response when administered orally or intranasally (Gregoriadis, *Immunol.Today*, 11:89, 1990; van Rooijen, *et al.*, *Immunological Adjuvants and Vaccines*, Gregoriadis, G., Allison, A.C., Poste G. (eds), Plenum Press, pp 95-106, 1989; Pierce, *et al.*, *Infect.Immunol.* 44:469, 1984). The potent adjuvant effect of liposomal delivery systems for antigen presentation is thought to be due, at least in part, to their ability to fuse with cell membranes and deliver their contents directly to intracellular antigen processing systems.

The immunosuppressed host is especially susceptible to infection at mucosal surfaces. Immunosuppression can be caused, for example, by chemotherapy, trauma, burns, and hemorrhage, especially following injury. Blood loss is a central factor in the pathophysiologic instability that follows injury, and has been shown to increase susceptibility and mortality to infection. Although blood loss does not result in changes in the absolute or relative numbers of T or B cell subsets (i.e., CD3+, CD4+, CD8+, Ig+ or B220+) in spleen, lymph nodes, bone marrow, thymus, or lungs, widespread changes in T and B cell function have been described after hemorrhage. Hemorrhage produces multiple abnormalities in immunologic

function, including alterations in cytokine (IL-1, IL-2, IL-3, IL-5, γ-IFN) release (Abraham, *et al., Circ.Shock*, 25:33, 1988; Abraham, *et al., J.Immunol.*, 142:899, 1989), decreases in mitogen induced T cell proliferation (Abraham, *et al., J.Immunol.*, 142:899, 1989), reduction in IL-2 receptor expression (Abraham, *et al., J.Immunol.*, 142:899, 1989; Stephan, *et al., Arch.Surg.*, 122:62, 1987) and changes in splenic (Abraham, *et al., Cell.Immunol.*, 122:208, 1989), intestinal (Abraham, *et al., Cell Immunol.*, 128:165, 1990), and pulmonary (Robinson, *et al., J.Immunol.*, 145:3734, 1990), B cell repertoires.

Studies (Esrig, *et al., Rev.Surg.*, 33:431, 1976; Stephan, *et al., Arch.Surg.*, 122:62, 1987) have shown increased susceptibility to infection following hemorrhage. Unfortunately, the results from those studies are difficult to interpret both because the models for hemorrhage were severely immunosuppressive, even if no blood were withdrawn, and the infections produced were not closely related to those seen clinically. In particular, the previously described models for hemorrhage involve the placement of arterial catheters into animals anesthetized for prolonged periods. Such animal preparations, even without any blood withdrawal, result in greater than 70% decreases in mitogen induced lymphocyte proliferation (Stephan, *et al., Arch.Surg.*, 122:62, 1987), inflammatory response (Abraham, *et al., Arch.Surg.*, 119:1154, 1984), as well as IL-1 (Ayala, *et al., Immunology*, 70:33, 1990) and IL-2 production (Stephan, *et al., Surg.Forum*, 37:73, 1986).

Many of the pathogenic organisms associated with infection of mucosal surfaces, such as the respiratory tract and gut, are bacteria where often the most desirable antigen for purposes of protective immunity is a polysaccharide. Unfortunately, polysaccharide antigens are generally poor immunogens even when administered systemically (Schreiber, *et al., J.Immunol.*, 146:188, 1991). This is especially true where the host is immunocompromised. Enhancement of the systemic immune response to

polysaccharide antigens has been achieved through coupling to protein carriers and adjuvants, or through anti-idiotypic manipulation (Schreiber, *et al., J.Immunol.*, 144:1023, 1990. In contrast, little information is available concerning the effects of immunization with bacterial polysaccharides on the antigen-specific mucosal immune response. In previous studies (Abraham, *et al.*, Vaccine, In press), oral immunization with the bacterial polysaccharide antigens levan or *Pseudomonas aeruginosa* polysaccharide type 1 coadministered with cholera toxin, as an adjuvant, was found to produce increased antigen specific pulmonary sIgA titers. However, large amounts of antigen (i.e., 1 mg) as well as the use of a toxic adjuvant were required in order to enhance the pulmonary plasma cell response.

As a consequence, there is considerable need for an effective non-toxic method of immunizing mucosal surfaces against various bacterial infections. The present invention provides such method by combining a bacterial polysaccharide antigen with a liposomal encapsulation carrier.

SUMMARY OF THE INVENTION

Recognizing the role that pathogenic bacteria play in mucosal infections and the severe therapeutic limitations of existing immunization techniques, the inventors produced and evaluated novel vaccines in an effort to develop a more effective method of immunization. These efforts have culminated in the development of a new method of immunization which is particularly effective against bacteria which infect or invade the host through mucosal surfaces.

This method was developed utilizing compositions which comprise a bacterial polysaccharide antigen encapsulated in a liposome. Surprisingly, these compositions and this method enables the host to mount a significant antigen-specific secretory immune response to the encapsulated bacterial polysaccharide antigen while minimizing adverse reactions to the vaccine.

The method of the invention is enhanced by use of novel liposomal compositions disclosed herein. These compositions comprise a bacterial polysaccharide antigen encapsulated in a liposome and further including an adjuvant. The adjuvant can be compartmentalized with the antigen, inserted into the liposomal membrane, or both.

DESCRIPTION OF THE DRAWINGS

FIGURE 1. Anti-levan sIgA titers in lung lavages of mice immunized intranasally with liposomes containing 38 mg levan. Mice were immunized at the indicated times following 30% blood volume hemorrhage, with resuscitation 1 hour later. Control (C) mice were immunized 3 days following ether anesthesia and cardiac puncture, but without blood withdrawal. Results are shown as OD_{450} ± SEM for undiluted lavage specimens, since the maximal OD was consistently found when the samples were tested without dilution (*$p<0.05$ versus Control).

FIGURE 2. Effects of hemorrhage with resuscitation on the numbers of levan-specific pulmonary plasma cells. Mice were immunized at the indicated times following 30% blood volume hemorrhage, with resuscitation 1 hour later. Control (C) mice were immunized 3 days after ether anesthesia and cardiac puncture, but without blood withdrawal. Results are shown as the number of total and IgA producing levan-specific plasma cells per set of lungs ± SEM. (*$p<0.05$ and **$p<0.01$ versus Control).

FIGURE 3. Survival of hemorrhaged mice intranasally immunized with 50 µl of empty liposomes (Control), liposomes containing 25 µg *Pseudomonas aeruginosa* polysaccharide type 1 (PsA), or liposomes containing 25 µg *Pseudomonas aeruginosa* polysaccharide type 1 and 0.2 µg (10 µg/kg) IL-2 (PsA + IL-2). The mice were immunized 2 hr following 30% blood volume hemorrhage, and then were inoculated intratracheally with 5×10^7 cfu *Pseudomonas aeruginosa* Fisher-Devlin immunotype 1 (strain 15921) 4 days following blood loss. Each group consisted of 11 animals. At 10 days following the induction of pneumonia, the mortality of mice intranasally immunized with liposomes containing *Pseudomonas aeruginosa* polysaccharide and IL-2 was significantly reduced ($p<0.005$) compared to the groups of animals immunized with empty liposomes or immunized with liposomes containing *Pseudomonas aeruginosa* polysaccharide, but no IL-2.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The inventors have devised compositions and methods for immunizing an animal to induce a mucosal immune response to a bacterial polysaccharide antigen which represents a significant improvement over the prior art methods intended to accomplish this effect. The present invention comprises the administration, to an animal which has, or is at risk of having, an infectious disease which occurs via contact between the infectious organism and a mucosal surface.

In the present invention, liposomes are used to encapsulate the bacterial polysaccharide antigen. When phospholipids are gently dispersed in aqueous media, they swell, hydrate, and spontaneously form multilamellar concentric bilayer vesicles with layers of aqueous media separating the lipid bilayer. Such systems are usually referred to as multilamellar liposomes or multilamellar vesicles (MLV's) and have diameters ranging from about 100nm to about 4μm. When MLV's are sonicated, small unilamellar vesicles (SUV's) with diameters in the range of from about 20 to about 50 nm are formed, which contain an aqueous solution in the core of the SUV.

The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used.

Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingolipids, cerebrosides, and gangliosides. Particularly useful are diacylphosphatidylglycerols, where the lipid moiety contains from 14-18 carbon atoms, particularly from 16-18 carbon atoms, and are saturated. Illustrative phospholipids include egg

phosphatidylcholine, dipalmitoylphosphatidylcholine, and distearoylphosphatidylcholine.

In preparing liposomes containing the bacterial polysaccharide antigen, such variables as the efficiency of antigen encapsulation, lability of the antigen, homogeneity, and size of the resulting population of liposomes, antigen-to-lipid ratio, permeability and instability of the preparation, and pharmaceutical acceptability of the formulation should be considered. (Szoka, *et al., Annual Reviews of Biophysics and Bio-engineering*, 9:467, 1980; Deamer, *et al.*, in *Liposomes, Marcel Dekker,* New York, 1983, 27; Hope, *et al., Chem.Phys.Lipids*, 40:89, 1986).

The invention is particularly useful in inducing an immune response in an animal, such as a human, which has been immunocompromised. The term "immunocompromised" denotes an animal having an immune system which is not functioning normally. Examples of conditions which can cause an animal to become immunocompromised include chemotherapy, irradiation, age, and physical trauma, such as resulting from shock, severe hemorrhage blood loss, or burns.

Many organisms, especially bacteria, utilize mucosal surfaces as sites of infection or initial invasion. In the present invention, the liposomal vaccine is able to induce a secretory immune response at the mucosal surface in order to ameliorate the pathogenic effect of these various organisms. Because of the interrelatedness of the various limbs of the mucosal immune system, it is possible to vaccinate the animal at one mucosal site and produce a secretory immune response at a different site. Examples of tissues which can be immunized and/or can produce a secretory immune response include, the respiratory tract, the gastro-intestinal tract, and the urinary tract, as well as glands such as the mammary glands, salivary glands and tear ducts. Thus, it is possible to induce a secretory immune

response against a gastro-intestinal pathogen by immunizing intranasally. Alternatively, it is possible to induce a secretory immune response in the respiratory tract by immunization of the gut, such as the Peyer's patches or lamina propria.

The term "immunogenically effective amount" as used in the invention denotes that amount of polysaccharide antigen which is necessary to induce the animal to produce antibodies which will bind to epitopes present on the polysaccharide antigen.

The method and compositions of the invention are especially useful in allowing immunization of bacterial polysaccharide antigens. Among the various bacterial polysaccharide antigens which are preferred are those derived from species or strains of *Aerobacter, Klebsiella, Proteus, Salmonella, Shigella, Campylobacter, Pseudomonas,* and *Streptococcus*. Especially preferred are bacterial polysaccharides derived from strains of *Pseudomonas aeruginosa* and *Streptococcus pneumoniae.* These bacterial polysaccharide antigens can be derived from a single strain or serotype or they may be polyvalent, i.e., a pool of antigens from various serotypes. The use of a polyvalent bacterial polysaccharide antigenic mixture is especially useful for organisms such as *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* where different serotypes are implicated in the etiology of disease. Techniques for the preparation of polysaccharide antigens from various bacteria are well known to those of skill in the art or can be readily ascertained without undue experimentation.

It is also possible for the liposomal preparations containing the bacterial polysaccharide antigen to include an adjuvant. Adjuvants are substances that can be used to nonspecifically augment a specific immune response. Normally, the adjuvant and the antigen are mixed prior to presentation to the immune system, or presented separately, but into the same site of the

animal being immunized. Adjuvants can be loosely divided into several groups based on their composition. These groups include oil adjuvants (for example, Freund's Complete and Incomplete), mineral salts (for example, $AlK(SO_4)_2$, $AlNa(SO_4)_2$, $AlNH_4(SO_4)$, silica, alum, $Al(OH)_3$, $Ca_3(PO_4)_2$, kaolin, and carbon) polynucleotides (for example, poly IC and poly AU acids), and certain natural substances (for example, wax D from *Mycobacterium tuberculosis*, B subunit of cholera toxin, glutaraldehyde treated cholera toxin, as well as substances found in *Corynebacterium parvum*, *Bordetella pertussis*, and members of the genus *Brucella*).

Other adjuvants which can also be incorporated in the liposomal preparations include immunomodulators and other biological response modifiers. The term "biological response modifiers" is meant to encompass substances which are involved in modifying the immune response in a manner which enhances the immune response to the polysaccharide antigen. Examples of biological response modifiers include lymphokines such as the interleukins, macrophage activating factors, migration inhibition factor, colony stimulating factors and the interferons. Preferred interleukins are those which display a direct affect on *in vitro* B-cell function, such as proliferation, antibody production, or activation. Especially preferred are IL-2 and IL-6. Other biological response modifiers are known, or readily ascertainable, by those of skill in the art.

The liposomal preparations of the invention can also be modified to contain an adjuvant in the membrane of the liposome. Preferred compounds are those characterized as lipoidal amines which are disclosed in Chang, *et al.*, *Arthritis and Rheumatism*, 21:169, 1978 and Chang, *et al.*, *Arthritis and Rheumatism*, 23:62, 1980, which are herein incorporated by reference. Especially preferred among the lipoidal amines is avridine (MN-dioctadecyl-N^1, N^1-2-hydroxymethyl-propane-diamine). Liposomes containing avridine can encapsulate multiple antigens, and the presence of avridine in the

liposomal membrane potentiates the adjuvant effect of the liposome, so that a greater mucosal immune response can be achieved after the administration of antigen in avridine containing liposomes. Avridine and bacterial polysaccharide containing liposomes can be prepared by the detergent dialysis technique (as modified by Philippot, *et al.*, *Biochem Biophys Acta*, 734:137, 1983) to produce large unilamellar liposomes, capable of encapsulating large amounts of antigen. Other techniques for liposome preparation such as the dehydration-rehydration modification (Kirby and Gregoriadis, *Biotechnology*, 2:979, 1984) also yield high encapsulation percentages. Regardless of the adjuvant which is utilized according to the invention, it is possible for the adjuvant to be present within the liposome itself, i.e., compartmentalized with the polysaccharide antigen, in the membrane of the liposome vesicle, or both. Those of skill in the art are familiar with techniques for the inclusion of adjuvant within the liposome (for example, complexed to the bacterial polysaccharide antigen) or within the liposomal membrane.

Many different techniques exist for the timing of the immunizations when a multiple immunization regimen is utilized. It is possible to use the liposomal preparation of the invention more than once to increase the levels of the ameliorating secretory immune response.

Generally, the dosage of polysaccharide antigen administered to an animal will vary depending upon such factors as age, condition, sex, and extent of the disease, if any, and other variables which can be adjusted by one of ordinary skill in the art. However, it is preferred, at least in instances where the animal has been subject to an immunosuppressive event due to trauma, such as severe blood loss, to administer the immunization within 24-48 hours, preferably within 24 hours.

The antigenic preparations of the invention can be administered as either single or multiple dosages and can vary from 10 μg/ml to 10,000 μg/ml, more preferably from 50 μg/ml to 5,000 μg/ml antigen per dose, most preferably 100 μg/ml to 600 μg/ml antigen per dose. Generally, those dosages which elicit the highest levels of sIgA (secretory immune response) to the bacterial polysaccharide antigen are preferred.

When a biological response modifier, such as an interleukin is included with the antigen in the liposome, dosages of modifier can vary from about 0.001 μg/ml to about 1000 μg/ml, preferably from about 0.01 μg/ml to about 200 μg/ml, most preferably from about 0.05 μg/ml to about 50 μg/ml.

Having now generally described the invention, a more complete understanding can be obtained by reference to the following specific examples. These examples are provided for the purpose of illustration only and are not intended to be limiting unless otherwise specified.

EXAMPLE 1

IMMUNOGENIC STUDIES OF LIPOSOME ENCAPSULATED BACTERIAL POLYSACCHARIDE ANTIGENS

Studies were done in 8-12 week old male BALB/C mice (Jackson Labs, Bar Harbor, ME) to determine the secretory immune response to bacterial antigen encapsulated in liposomes.

Liposomes were prepared by the detergent dialysis technique, as modified by Philippot, *et al.* (Biochem Biophys Acta, 734:137, 1983) to produce large unilamellar liposomes, capable of encapsulating high amounts of antigen. Cholesterol, phophatidylserine, and phosphatidylcholine (8 μM each) were

combined, dried to a film under N_2, and then placed in a lyophilizer for another 60 minutes. The bacterial polysaccharide to be encapsulated was suspended in 0.5 ml of buffer (150 mM NaCl, 10 mM HEPES (GIBCO), 1 mM EDTA, pH 7.4) and added to the dry lipids. C^{14}-labelled inulin (20 μ1), to monitor encapsulation efficiency, was added to the suspension. After 30 minutes, 0.24 ml of 1 M octylglucoside (Calbiochem) was added to the lipid and antigen mixture, and shaken vigorously. The sample was transferred into dialysis tubing (molecular weight cutoff 3500), and dialyzed against 100 ml of buffer containing 2.4 g SM-2 Biobeads (Biorad). After 24 hours, the liposome preparation was placed on the A5M (Biorad) column, and the liposome fraction (in the void volume) collected. Encapsulation efficiency was determined by comparing the C^{14} counts in the initial lipid suspension to that in the final liposome fractions. In general, encapsulation efficiencies of approximately 40% (levan and *Pseudomonas aeruginosa* type 1) and 10% *Streptococcus pneumoniae* type 3 polysaccharide (PPS 3) were obtained. The average size of the unilamellar liposomes was 995 ± 80 nm, as determined by dynamic laser light scattering using a Coulter NS4D instrument.

Animals were immunized intranasally by applying 0.1 ml of liposomal suspension to the nares and allowing this to be inhaled. Antigens tested were levan (38 μg) from *Aerobacter levanicus* (Sigma, St. Louis, MO), polysaccharide type 1 (33 μg) from *Pseudomonas aeruginosa*, and pneumococcal polysaccharide type 3 (33 μg) from *Streptococcus pneumoniae*.

Pulmonary lavages were obtained by pooling bronchoalveolar washings produced by injecting 0.5 ml of phosphate buffered saline (PBS) 3 times into the trachea and lungs. In general, a final volume of approximately 0.4 ml was obtained. The washings were centrifuged to remove cellular debris and stored at -20°C until tested.

Pulmonary lavages and serum were tested for production of antibody against the bacterial polysaccharide antigens using an ELISA technique (Portnoi, *et al., Eur.J.Immunol.*, 18:571, 1988). The 96 well ELISA plates (Costar, 3590, Cambridge, MA) were coated overnight with antigen (10 μg/ml) in 0.05 M phosphate buffer, pH 8. After washing the plates with PBS, the wells were saturated with PBS containing 1% ovalbumin for 30 minutes at 37°C. After further washing with PBS, 50 μl of pulmonary lavage fluid or serum in serial 2-fold dilutions was transferred to the ELISA plates, and the plates incubated for 2 hours at room temperature. After washing, peroxidase-coupled goat anti-mouse antibody was added to each well. After a further 1 hour incubation, the bound antibody was revealed with chromogen containing H_2O_2 and orthophenyldiamine. The reaction was stopped 20 minutes later by addition of 50 μl of 10% SDS to each well and the OD measured at 450 nm in a photometer (MR 600, Dynatech Instruments, Torrance, CA) with a 410 nm correction filter.

TABLE 1

ORGANISM	ANTIGEN	GROUP	
		CONTROL	IMMUNIZED
Aerobacter levanicus	levan	0.046 ± 0.017	0.126 ± 0.023
Pseudomonas aeruginosa	type 1	0.255 ± 0.061	0.660 ± 0.051
Streptococcus pneumoniae	PPS 3	0.127 ± 0.044	0.293 ± 0.115

The data in Table 1 shows that animals vaccinated via the mucosal immune system produce significant titers of sIgA specific for a given bacterial polysaccharide antigen when the antigen is presented in the form of a vaccine which utilizes a liposome carrier system to encapsulate the bacterial polysaccharide antigen.

EXAMPLE 2

IMMUNIZATION OF IMMUNOCOMPROMISED ANIMALS

The effect of immunization with liposomally encapsulated bacterial polysaccharide antigen was studied in immunocompromised animals. Immunocompromization was induced in mice using the murine hemorrhagic model (Abraham, *et al., J.Immunol.*, 142:899, 1989). Briefly, mice were anesthetized with inhaled ether after being placed into a covered beaker. Cardiac puncture was performed with a 30-gauge needle and 30% of the calculated blood volume (approximately 0.55 ml for a 20 g mouse) was withdrawn over a 60-second period. The blood was collected into a syringe containing 100 U heparin in 0.1 ml, and then was kept at 37°C for 1 hour until reinfusion into the unanesthetized mouse through a retroorbital plexus injection. Using this method, it was possible to resuscitate all hemorrhaged mice without complication. The mouse then was allowed to recuperate in its cage. The total period of ether anesthesia was less than 2 minutes in all cases. The mortality rate with this hemorrhage protocol is approximately 12%, with all deaths occurring during the first 24 hours post hemorrhage, and most deaths occurring within the 1 hour post hemorrhage.

With this hemorrhage model, ether anesthesia and cardiac puncture without blood withdrawal result in no changes in mitogen-induced lymphocyte proliferation, IL-2R expression, phenotypic characteristics (CD3, CD4, CD8, B220, μ, Ly-1 expression) of B or T lymphocytes, lymphokine (IL-2, IL-3, IL-5,

γ-IFN) release, or splenic, intestinal, or pulmonary B cell clonal precursor frequencies (Robinson, *et al., J.Immunol.*, 145:3734, 1990). In particular, previous experiments had found no alterations in the numbers or frequencies of pulmonary B cell clonal precursors specific for levan or *Pseudomonas aeruginosa* polysaccharide type 1 in mice after ether anesthesia and cardiac puncture, but no blood withdrawal (Robinson, *et al., J.Immunol.*, 145:3734, 1990). Similarly, no evidence of hemothorax, bleeding into the pericardial space, lung, or cardiac contusion has been found in surviving mice with this method of hemorrhage (Abraham, *et al., J.Immunol.*, 142:899, 1989). Animal selection, immunization, and ELISA techniques were performed as described in Example 1.

Intraparenchymal lung lymphocytes were isolated as previously described (Abraham, *et al., J.Immunol.*, 144:2117, 1990). In brief, 3 mice were used for each experiment, and the lung cells pooled for analysis. After the mouse was killed by cervical dislocation, the chest was opened and the lung vascular bed was flushed by injecting 3-5 ml of chilled (4°C) PBS into the right ventricle. The lungs were then excised, avoiding the peritracheal lymph nodes, and washed twice in RPMI 1640 (GIBCO). The lungs were minced finely, and the tissue pieces placed in RPMI 1640 with 5% FCS (GIBCO), penicillin/streptomycin, 10 mM HEPES, 50 μM 2-ME (GIBCO), 20 mM L-glutamine (GIBCO), containing 20 U/ml collagenase (SIGMA), and 1 μg/ml DNase (SIGMA). Following incubation for 60 minutes at 37°C any remaining intact tissue was disrupted by passage through a 21 gauge needle. Tissue fragments and the majority of dead cells were removed by rapid filtration through a glass wool column, and cells collected by centrifugation. The cell pellet was suspended in 4 ml of 40% Percoll (Pharmacia, Uppsala, Sweden) and layered onto 4 ml of 80% Percoll. After centrifugation at 600 g for 20 minutes at 15°C the cells at the interface were collected, washed in RPMI 1640, and counted. Viability was consistently greater than 98% as determined by trypan blue exclusion.

Detection of total numbers and levan-specific plasma cells in the lung was performed using a technique similar to that described by Sedgwick and Holt (Sedgwick, *et al., J.Immunol. Methods*, 57:301, 1983. Briefly, 96 well flat bottom ELISA plates were coated by overnight incubation at 4°C with levan (10 μg/ml) in 50 μl/well 0.05 M potassium phosphate buffer, pH 8. The plates then were washed with PBS, and the wells saturated with PBS containing 1% gelatin for 1 hour at 37°C. After further washing with PBS, the wells were seeded with a titration of intraparenchymal lung lymphocytes from immunized or unimmunized mice in 100 μl RPMI containing glutamine, 2-ME, 10 mM HEPES, penicillin/streptomycin, and 1% FCS. The number of cells per well was adjusted to be from 10^2 to 10^6.

The plates then were incubated at 37°C in 5% CO_2 for 5 hours. The cells were removed by flicking the plate, followed by lysis with 0.05% Tween 20 in H_2O. After washing with PBS containing 0.05% Tween, goat anti-mouse total Ig coupled with biotin was added to the wells in PBS 1% gelatin, and the plates left at 4°C overnight. The plates then were washed in PBS, 0.05% Tween, streptavidin coupled with alkaline phosphatase added to the wells. After a 1 hour incubation at 37°C, the plates were washed and the revealing substrate, 2.3 mM 5-bromo-4-chloro-3-indolyl phosphate (Sigma, St. Louis, MO) diluted in 2-amino-2-methyl-l-propanol (Sigma, St. Louis, MO), containing 0.75% agarose was added to each well. After a 1 to 2 hour incubation period at 37°C, the antibody secreting cells were revealed as blue spots which could be counted. The dilution of cells producing 20 to 40 spots/well was used to quantitate the total number of antibody-specific B cells/sample. The sensitivity and specificity of this assay has been documented in Ag-inhibition tests and in studies involving Ag-specific hybridoma cell lines (Klinman, *et al., J.Immunol.*, 141:801, 1988; Sedgwick, *et al., J.Immunol. Methods*, 87:37, 1986.

Pneumonia was induced using Fisher-Devlin immunotype 1 strain 15921 of *Pseudomonas aeruginosa* obtained from Dr. Gerald Pier, Harvard University, Boston, MA. After fresh plating on tryptic soy agar plates, the bacteria were transferred to tryptic soy broth and incubated at 37°C until a concentration of 10^9 cfu was achieved. The bacteria were centrifuged, washed twice in chilled PBS, then resuspended to a concentration of 5×10^8, 10^8, 2×10^9, or 3×10^9 cfu/ml in PBS. Mice were anesthetized with pentobarbital (50 mg/kg) i.p., vertically suspended, and 40 µl of the bacterial suspension introduced into the trachea using a blunt 22 gauge needle passed through the mouth. The mice were returned to their cages and permitted free access to food and water. Mortality was assessed twice each day. Mice were observed for a 7 day period, but no mortality was found after day 5.

In experiments examining antigen-specific pulmonary sIgA titers and pulmonary plasma cells, 2 groups of mice (each group consisting of 3 mice) were examined for each experimental condition. ELISA was performed on pulmonary lavages from each mouse (6 lavages examined for each condition). For the ELISA Spot assay, pooled lung lymphocytes were isolated from each group of 3 mice. The number of antigen-specific plasma cells per set of lungs was calculated by multiplying the number of antigen-specific spots per 10^6 lung lymphocytes, as determined by the ELISA Spot Assay, by the total number of cells isolated per set of lungs.

Data are presented as mean ± standard error (SEM) for each experimental group. Comparisons between means of groups were performed by the Student t test for differences between 2 groups or by using one way analysis of variance for examining differences for experiments with more than 2 groups. Survival data were analyzed by Chi-square and Fisher's Exact analysis. A p value less than 0.05 was considered to be significant.

A. RESULTS OF INTRANASAL IMMUNIZATION

Because of the existence of a common mucosal immune system (Mestecky, *et al., J.Clin.Immunol.*, 7:265, 1987), where secretory antibody responses generated at one mucosal surface subsequently are found at other mucosal sites, mice were intranasally immunized with increasing amounts (1, 5, 10, and 38 μg) of the bacterial polysaccharide antigen levan encapsulated in liposomes. One week later the animals were sacrificed, the lungs lavaged as described above, and the bacterial antigen specific sIgA titers measured by ELISA. In addition, lung lymphocytes were isolated and the number of bacterial antigen specific plasma cells per set of lungs was enumerated using the ELISA Spot assay. Serum also was obtained, and anti-levan titers measured by ELISA.

In unimmunized mice and in animals intranasally immunized with between 1 and 10 μg of liposome encapsulated levan, no levan-specific plasma cells were found among lung lymphocytes. In contrast, 33 ± 5 levan-specific plasma cells per set of lungs were present following intranasal immunization with 38 μg of the levan containing liposomes. Similarly, increases in the levan specific sIgA titers were found only in mice immunized with 38 μg of the liposome encapsulated levan (OD_{450}=0.126 ± 0.023 versus 0.046 ± 0.017 in unimmunized mice). No changes in serum anti-levan titers were found in any group of intranasally immunized mice as compared with the unimmunized group.

B. EFFECTS OF HEMORRHAGE ON PULMONARY PLASMA CELL RESPONSE

In order to examine the effects of hemorrhage induced immunosuppression on the pulmonary secretory antibody response to bacterial antigens, 30% of mouse blood volume was removed, then returned 1 hour later.

Liposomes containing 38 μg levan were administered intranasally at predetermined timepoints post hemorrhage. One week following immunization, lung lavages and pulmonary lymphocytes were collected for analysis of levan-specific sIgA titers and plasma cell numbers. Serum also was collected 1 week post immunization, and anti-levan titers measured. The results of these experiments are shown in FIGURES 1 and 2.

Anti-levan sIgA titers in lung lavages from mice immunized 3 days after blood loss were not different from those in normal or control animals. However, anti-levan sIgA titers were significantly decreased in mice immunized 7 and 14 days following hemorrhage (FIGURE 1). Reduced numbers of levan-specific pulmonary plasma cells also were found after hemorrhage and resuscitation, but with kinetics somewhat different from that present for anti-levan sIgA titers in lung lavages. More than 70% decreases in the numbers of levan-specific plasma cells, both total and IgA producing, were present between 1 and 7 days after hemorrhage, with return to prehemorrhage values present 14 days after blood loss (FIGURE 2). Essentially no levan-specific pulmonary plasma cells were found when immunization was performed 3 days post hemorrhage.

Hemorrhage, rather than other components of the model, was responsible for alterations in the anti-levan response among pulmonary plasma cells. Immunization of mice 3 days following ether anesthesia and cardiac puncture, but without blood withdrawal (control), resulted in no significant changes in either anti-levan sIgA titers in lung lavages (OD_{450} 0.115 ± 0.009 in controls versus 0.126 ± 0.023 in normals) nor in numbers of levan-specific pulmonary plasma cells (35 ± 7 in controls versus 33 ± 5 in normals). No changes in serum anti-levan titers as compared to normal, unhemorrhaged mice were found in control animals or at any time point following blood loss.

C. EFFECT OF HEMORRHAGE ON PNEUMONIA SURVIVAL

The previous experiments demonstrated that the pulmonary secretory antibody and plasma cell responses to a bacterial polysaccharide antigen were decreased between 1 and 14 days following hemorrhage and resuscitation. Antibodies directed against bacterial polysaccharides, including *Pseudomonas aeruginosa* polysaccharides, are protective against infection with the organisms from which these antigens were isolated (Pier, *et al., Infect.Immunol.*, 22:919, 1978; Stein, *et al., J.Exp.Med.*, 160:1001, 1984). The present results therefore suggested that pulmonary resistance to infection might be reduced at this time point following blood loss. In order to examine this hypothesis, experimental *Pseudomonas aeruginosa* pneumonia was induced in normal, hemorrhaged, and hemorrhaged and resuscitated mice using intratracheal injection of increasing numbers of *Pseudomonas aeruginosa* Fisher-Devlin immunotype 1 organisms.

In unmanipulated mice or mice subjected to ether anesthesia and cardiac puncture, but no blood withdrawal, no mortality was found when groups of mice (n = 6) were given 2×10^7 or 4×10^7 organisms. However, when mice (n = 6) were given 8×10^7 organisms intratracheally, the mortality rate was 83%, and when 12×10^7 organisms were injected, the mortality rate was 100%. All mice died between 24 and 96 hours after being infected. Histologic examination of the lungs in mice having died after intratracheal introduction of *Pseudomonas aeruginosa* organisms showed changes consistent with an acute and consolidative pneumonia.

Next, mice (n = 19) were bled 30% of their total blood volume. In one group of hemorrhaged mice (n = 7), the removed blood was reinfused 1 hour following hemorrhage. The remaining mice (n = 12) were left unresuscitated. A control group (n = 13) of mice subjected to ether anesthesia and cardia puncture, but no blood withdrawal was included.

After 4 days, 2×10^7 *Pseudomonas aeruginosa* organisms were inoculated into the trachea of each mouse.

TABLE 2

CONDITION	NUMBER DIED	NUMBER SURVIVED	% MORTALITY
Control	0	13	0
Hemorrhage	8	4	67[a]
Hemorrhage and Resuscitation	4	3	57[a]

[a] $p<0.01$ vs. Control

No animals in the control group died following infection. In contrast, the mortality rate was 67% in the mice bled 4 days prior to infection, and was 57% in the hemorrhaged and resuscitated mice (Table 2). The mortality rate in hemorrhaged animals which had been resuscitated was not significantly different from that in the hemorrhaged, but unresuscitated group.

Another experiment was done to study the effect of immunization on survival of animals which were immunosuppressed by hemorrhage. In this study, both control and immunized animals were hemorrhaged and resuscitated as described above. The 9 animals in the immunized group were vaccinated using *Pseudomonas aeruginosa* type number 1 polysaccharide (33 μg) encapsulated in liposomes 1 hour after hemorrhage and resuscitation. Both groups of animals were exposed to 2×10^7 viable cells of *Pseudomonas aeruginosa* 96 hours after hemorrhage and resuscitation. The results of this study are shown in Table 3.

TABLE 3

CONDITION	N	NUMBER DIED	NUMBER SURVIVED	% MORTALITY
Unimmunized	13	13	0	100
Immunized	9	6	3	67*

* $p<0.05$

As shown in Table 3, immunized animals showed a significantly increased level of survival (33%) as compared to animals in the unimmunized group. Thus, intranasal immunization with liposomally encapsulated bacterial polysaccharide antigen can significantly enhance the survival of immunosuppressed animals at risk of pathogenic or nosocomial infection or colonization.

The experimental results illustrated in Tables 2 and 3 demonstrate that hemorrhage, even when followed by resuscitation, results in marked and long lasting depression in pulmonary antibacterial B cell function. Reduced numbers of pulmonary plasma cells producing antibody against the immunizing bacterial polysaccharide antigen were found between 1 and 14 days following blood loss, and titers of bacterial antigen specific sIgA were decreased for more than 2 weeks after hemorrhage. The difference in post hemorrhage kinetics between anti-levan sIgA and levan-specific plasma cells is not surprising. Because secreted antibodies can persist at mucosal surfaces for several days, antigen-specific antibody titers would not be expected to immediately reflect changes in plasma cell numbers, but rather to follow the pattern of plasma cell alteration in a delayed fashion. The significance of these abnormalities in pulmonary B cell function was

demonstrated by an increased susceptibility to *Pseudomonas aeruginosa* pneumonia at a time point 4 days following hemorrhage, when bacterial antigen-specific pulmonary plasma cell numbers were at their lowest point.

Previous studies by the inventors had shown that hemorrhage without resuscitation resulted in marked decreases in the numbers and percentages of pulmonary, intestinal, and splenic small resting B cells (clonal precursors) committed to the production of antibodies to bacterial antigens. Because plasma cells develop from B cell clonal precursors, alterations in clonal precursor repertoires are frequently reflected by parallel changes in antigen-specific plasma cell frequencies. This situation following unresuscitated hemorrhage had been previously demonstrated, where decreases in the frequency of bacterial antigen-specific splenic and intestinal clonal precursors were reflected by a similar decrease in frequency of antigen-specific plasma cells from these anatomic sites (Abraham, *et al., Cell Immunol.*, 122:208, 1989; Abraham, *et al., Cell Immunol.* 128:165, 1990). In hemorrhaged mice, decreased numbers of pulmonary B cell clonal precursors committed to producing antibody to the bacterial antigens levan and *Pseudomonas aeruginosa* polysaccharide type 1 occur between 3 and 10 days after blood loss (Robinson, *et al., J.Immunol.*, 145:3734, 1990). In the present experiments, the time course for post hemorrhage decreases of bacterial antigen-specific pulmonary plasma cell numbers and sIgA production is quite similar to that found among antigen-specific B cell clonal precursors, and therefore suggests that these hemorrhage induced alterations in pulmonary plasma cell number and function reflect alterations in the numbers and frequencies of bacterial antigen-specific B cell clonal precursors among the resting, small B cell population.

Decreased numbers of bacterial antigen-specific clonal precursors, able to be recruited into the plasma cell compartment, may be limiting in terms of host defense following exposure to a bacterial inoculum. The present

experiments suggest that such a mechanism may be responsible for the increased susceptibility to infection after blood loss, since increased mortality from *Pseudomonas aeruginosa* pneumonia following hemorrhage occurred at a time point associated with decreased numbers of bacterial polysaccharide antigen-specific B cell clonal precursors in the lung and a diminished ability to produce bacterial antigen-specific secretory antibodies. Animals resuscitated 1 hour following hemorrhage showed the same increased susceptibility to *Pseudomonas aeruginosa* pneumonia as did hemorrhaged, but unresuscitated mice. These results therefore suggest that abnormalities in immune response are rapidly and irreversibly induced by hemorrhage, and that resuscitation has limited effects in modifying these immunologic alterations.

Because of the ability of B and T cells to migrate from one mucosal surface to another, it was not surprising that the post hemorrhage time course of alteration in bacterial antigen-specific pulmonary plasma cells resembled that found previously (Abraham, *et al.*, *Cell Immunol.*, 128:165, 1990) among plasma cells isolated from intestinal lamina propria. In particular, in both mucosal sites, decreased numbers of bacterial antigen-specific plasma cells were found between 3 and 14 days post hemorrhage.

These studies show that increased bacterial antigen-specific antibody titers can be achieved in the lungs when the bacterial polysaccharide antigen levan was administered intranasally in a liposomal formulation. The amount of antigen encapsulated in liposomes and given intranasally, 38 μg, was approximately 30-fold less than that required with oral immunization to produce the same increase in numbers of levan-specific pulmonary plasma cells. As expected with a mucosal immunization technique, no change in serum anti-levan titers were found in immunized animals. Because liposomes are nontoxic and, as shown in this study, can act as potent adjuvants in enhancing the mucosal immune response to polysaccharide

antigens, they would be particularly useful in intranasal and oral vaccination formulations aimed at preventing nosocomial infection in immunocompromised, critically ill patients.

Nosocomial pneumonias are frequent following injury and other critical illnesses. The present experiments demonstrate that hemorrhage, even if resuscitated, results in alterations in pulmonary B cell function which are long lasting, and associated with increased susceptibility to infection at this mucosal surface. Interestingly, the disappearance of bacterial antigen-specific pulmonary B cell clonal precursors and plasma cells does not occur immediately post hemorrhage, so that significant numbers of B cell clonal precursors, able to be recruited into the plasma cell population and to produce bacterial antigen-specific antibody, are still present in the lungs during the first 24 hours post hemorrhage. These results suggest that immunization immediately post hemorrhage would be able to enhance pulmonary sIgA titers directed toward important bacterial antigens and might be able to increase resistance to pneumonia following hemorrhage or injury. The present experiments, showing that intranasal immunization with bacterial polysaccharides encapsulated in liposomes produce increased pulmonary bacterial antigen-specific sIgA titers, suggest that this approach may be useful in critically ill patients through enhancing the anti-bacterial mucosal immune response and thereby decreasing the incidence of nosocomial pneumonia, as well as morbidity and mortality associated with these respiratory tract infections.

EXAMPLE 3

IMMUNOGENIC EFFECT OF LIPOSOME ENCAPSULATED BACTERIAL POLYSACCHARIDE ANTIGEN AND INTERLEUKIN

The effect of encapsulation of IL-2 into liposomes containing bacterial polysaccharides on the antigen-specific secretory antibody response in the lungs was studied by intranasally immunizing mice with liposomes containing 25 μg of levan and increasing amounts of IL-2 (0 to 0.2 μg, i.e., 0 to 10 μg/kg). One week later, the animals were sacrificed, the lungs lavaged, and levan-specific Ig titers in lavage fluid measured by ELISA, as described above. In addition, lung lymphocytes were isolated and the number of total and levan-specific plasma cells per set of lungs enumerated using the ELISA spot assay (see EXAMPLE 2, Sedgwick, *et al., J. Immunol. Methods*, 57:301, 1983). Serum was obtained and anti-levan titers measured by ELISA.

TABLE 4

LEVAN-SPECIFIC IMMUNE RESPONSE IN PRESENCE OF IL-2

	LEVAN-SPECIFIC		
IL-2[a]	SECRETORY IgA TITERS[b]	TOTAL PULMONARY PLASMA CELLS	IgA PULMONARY PLASMA CELLS
0.0	0.172 ± 0.057	4.0 ± 0.5	3.6 ± 0.9
0.1	0.152 ± 0.029	2.4 ± 1.0	2.4 ± 1.0
1.0	0.290 ± 0.028	9.3 ± 1.0	8.3 ± 1.0
10.0	1.023 ± 0.169	333.0 ± 50.0	296.0 ± 43.0

[a] μg/kg, equivalent to 0, 0.002, 0.02, and 0.20 μg, respectively

[b] A_{450} ± SEM

As shown in TABLE 4, significant increases in anti-levan sIgA titers in lung lavages ($p<0.01$) were found in mice immunized with liposomes containing 10 μg/kg IL-2. There also was small, but significant ($p<0.01$) increase in pulmonary anti-levan titers in mice after immunization with liposomes containing 1 μg/kg IL-2 (OD_{450} 0.290 ± 0.029 versus 0.172 ± 0.057 in control mice immunized with liposomes containing levan, but no IL-2). No alternation in pulmonary anti-levan sIgA titers were found in mice immunized with a smaller dose of IL-2 (i.e., 0.1 μg/kg). In contrast to the large increases in antigen-specific sIgA titers in lung lavages from immunized mice, no changes in antigen-specific IgM or IgG titers occurred following intranasal immunization with IL-2 containing liposomes. Similarly, serum anti-levan titers were not significantly altered in any group of mice intranasally immunized with the IL-2 containing liposomes as compared to control, unimmunized mice.

Intranasal immunization with liposomes containing levan and 10 μg/kg IL-2 produced more than 80-fold increases in the number of levan-specific pulmonary plasma cells as compared to numbers present following vaccination with liposomes containing levan, but no IL-2. More than 90% of the levan-specific pulmonary plasma cells produced IgA. The number of levan-specific pulmonary plasma cells was increased by approximately 2-fold in animals immunized with liposomes containing 1 μg/kg IL-2. No alterations in the numbers of levan-specific plasma cells in lung digests were found in animals receiving lower doses of IL-2.

TABLE 5

EFFECT OF IL-2 ON FREQUENCY OF PLASMA CELLS

	PULMONARY PLASMA CELLS	
IL-2[a]	TOTAL	IgA
0.0	3712 ± 340	3392 ± 290
0.1	2460 ± 250	2280 ± 210
1.0	7885 ± 660	7470 ± 717
10.0	7408 ± 688	6698 ± 858

[a] μg/kg, equivalent to 0, 0.002, 0.02, and 0.20 μg, respectively

Immunization with liposomes containing IL-2 resulted in increases both in the relative frequency of levan-specific plasma cells in the lungs and in the total numbers of pulmonary plasma cells, producing antibody of all specificities. In animals immunized with liposomes containing levan alone, without IL-2, there were 4 ± 1 levan-specific plasma cells per 10^6 isolated lung lymphocytes, and 0.14% of all lung plasma cells were levan specific. In contrast, in mice immunized with liposomes containing 10 μg/kg IL-2, there were 93 ± 11 levan-specific plasma cells per 10^6 isolated lung lymphocytes, and these levan-specific plasma cells comprised approximately 4.1% of the lung plasma cell population. In addition, the total number of plasma cells isolated per set of lungs increased from 3712 ± 340 in mice immunized with liposomes containing levan alone to 7408 ± 688 in mice immunized with liposomes containing 10 μg/kg IL-2. Of note, the enhancing effects of liposome encapsulated IL-2 on pulmonary plasma cell numbers appear to

be somewhat distinct from the adjuvant effects of IL-2 on pulmonary plasma cell numbers appear to be somewhat distinct from the adjuvant effects of IL-2 on increasing antigen-specific plasma cell numbers. Intranasal immunization with liposomes containing 1 μg/kg IL-2 resulted in increases in total numbers of pulmonary plasma cells comparable to those seen when 10 μg/kg IL-2 was included in the liposomes (7885 ± 660 plasma cells/set of lungs for liposomes containing 1 μg/kg IL-2 versus 7408 ± 688 plasma cells/set of lungs for liposomes containing 10 μg/kg IL-2). In contrast, the number of levan-specific plasma cells per set of lungs increased by more than 35-fold, from 9 ± 1 to 333 ± 50 when the amount of IL-2 included in the liposomes was changed from 1 μg/kg to 10 μg/kg.

Because the previous experiments demonstrated potent adjuvant properties of liposome encapsulated IL-2 on bacterial antigen-specific secretory antibody response in the lungs, the possibility that inclusion of IL-2 into liposomes would permit use of a decreased dose of antigen, while still achieving enhancement of antigen-specific sIgA titers. To address this issue, liposomes containing 2.5 μg of levan and 0.2 μg IL-2 (i.e., 10 μg/kg) were prepared. No significant increase in levan-specific sIgA titers in pulmonary lavages as compared to unimmunized controls, and no levan-specific pulmonary plasma cells were found following immunization with this liposomal preparation, indicating that inclusion of IL-2 was unable to sufficiently enhance antibody production to compensate for a 10-fold reduction in the immunizing antigen.

In order to determine if the enhancement of bacterial polysaccharide antigen-specific secretory antibody response associated with liposomal encapsulation of IL-2 and levan could be achieved with other bacterial polysaccharides, mice were intranasally immunized with liposomes containing either *Pseudomonas aeruginosa* polysaccharide type 1 (25 μg)

alone or with 0.2 μg IL-2 (i.e., 10 μg/kg). The results of these studies are presented in TABLE 6.

TABLE 6

EFFECT OF IL-2 ON SECRETORY IgA RESPONSE TO *Pseudomonas aeruginosa* POLYSACCHARIDE (PsA)

GROUP	PsA	IL-2	SECRETORY IgA TITER[b]
1	–	–	0.466 ± 0.139
2	25 μg	–	0.584 ± 0.075
3	25 μg	0.2 μg[a]	0.941 ± 0.167

[a] 10 μg/kg

[b] A_{450} ± SEM

As was shown in EXAMPLE 1, intranasal immunization with liposomes containing *Pseudomonas aeruginosa* polysaccharide alone increased antigen-specific sIgA titers in pulmonary lavages, compared to those in animals immunized with empty liposomes (OD_{450} 0.4666 ± 0.139 for empty liposomes versus 0.584 ± 0.075 for liposomes containing *Pseudomonas aeruginosa* polysaccharide). Addition of IL-2 to liposomes produced significant increase ($p<0.01$) in anti-*Pseudomonas aeruginosa* polysaccharide sIgA titers when compared either to those found in animals immunized with empty liposomes or in mice immunized with liposomes containing *Pseudomonas aeruginosa* polysaccharide, but no IL-2.

Because IL-4 has been shown to affect B cell maturation and progression to antibody production, the possible adjuvant effects of encapsulation with IL-4 was investigated. However, no significant increases in anti-levan sIgA titers in lung lavages were found in mice intranasally immunized with liposomes containing 25 μg levan and 0.2 μg (.i.e., 10 μg/kg) IL-4 (OD_{450} 0.183 ± 0.075 in controls versus 0.144 ± 0.081 for mice immunized with IL-4 containing liposomes). Further, no levan-specific pulmonary plasma cells were found on ELISA Spot analysis in animals immunized with the IL-4 containing liposomes.

While not wanting to be bound to a particular theory, because of the relative predominance of Th2 cells at mucosal sites, it is possible that sufficient levels of IL-4, able to support B cell responses, are already present in these anatomic locations, and further increasing local IL-4 levels produces no additional effect. In addition, there is evidence that IL-4, while capable of stimulating the early steps in the B cell response, also has inhibitory properties on B cell proliferation, particularly that driven by IL-2. If this is the case *in vivo*, then providing increased local concentration of IL-4 may actually have an inhibitory effect on antibody production.

Further studies were done evaluating the effect of IL-2 containing liposomes to protect against pneumonia using the hemorrhage model previously described. In these experiments, mice were bled 30% blood volume, then 2 hr later intranasally administered liposomes containing either 25 μg *Pseudomonas aeruginosa* polysaccharide type 1 alone, or 25 μg *Pseudomonas aeruginosa* polysaccharide and 0.2 μg IL-2, or 25 μg levan and 0.2 μg IL-2. Four days later, the animals were infected intratracheally with *Pseudomonas aeruginosa.*

In unimmunized mice or mice immunized with liposomes containing levan and IL-2, the mortality rate was 100% following the induction of

Pseudomonas aeruginosa pneumonia (FIGURE 3). No significant improvement in survival was found in mice immunized with liposomes containing *Pseudomonas aeruginosa* polysaccharide, but no IL-2. In contrast, the mortality rate was reduced to 45% in mice intranasally immunized with liposomes containing *Pseudomonas aeruginosa* polysaccharide and 10 μg/kg IL-2 ($p<0.005$). Histopathologic studies showed acute consolidative and hemorrhagic pneumonia with the presence of gram negative rods in the lungs of both immunized and unimmunized mice which died following intratracheal introduction of bacteria.

Intranasal immunization of mice with liposomes containing IL-2 and *Pseudomonas aeruginosa* polysaccharide provided significant protection in a pneumonia model which is 100% lethal in unimmunized mice. Of note, vaccination with liposomes containing IL-2 and a bacterial polysaccharide (levan) derived from another organism (*Aerobacter levanicum*) was not effective against *Pseudomonas aeruginosa* pneumonia, demonstrating that the protection achieved by immunization with liposomes is not due to nonspecific enhancement of pulmonary immune response. The dose of bacteria used to produce pneumonia in the present experiments, 5×10^7 cfu, was more than twice as high as we have used previously, when mortality rates of 100% in unimmunized animals also were found. In previous studies, it was found that liposomally encapsulated *Pseudomonas aeruginosa* polysaccharide, without IL-2, was able to provide partial protection from mortality due to pneumonia. The lack of protection of this formulation in the present experiments is probably due to the higher dose of bacteria used to produce pneumonia and to the smaller amount of polysaccharide incorporated into liposomes (25 μg versus 33 μg used in previous studies).

In order to evaluate the effectiveness of other interleukins, studies similar to those described for IL-2 and IL-4 were performed using recombinant human IL-6 in combination with polysaccharide antigen. Results are shown in TABLE 7.

TABLE 7

EFFECT OF IL-6 ON LEVAN-SPECIFIC IMMUNE RESPONSE

Amount of IL-6[a] Administered	Levan-Specific Plasma Cells/Set of Lungs		% Levan-Specific Pulmonary Plasma Cells
	Total	I_0A	
0.0	9	8	0.13
0.1	29	19	2.04
1.0	106	96	1.89
5.0	61	41	1.77

[a] μg/kg, equivalent to 0, 0.002, 0.02, 0.10 μg, respectively

In each case, male BALB/c mice were immunized intranasally with 50 μl of suspension containing 25 μg levan and the above-noted amount of human recombinant IL-6. Five days later the lungs were digested, lung lymphocytes purified, and the number of levan-specific pulmonary plasma cells per set of lungs determined using the ELISA Spot assay as previously described. As seen here, the incorporation of IL-6 into liposomes resulted in marked increases in the number of levan-specific lung plasma cells, showing a potent adjuvant effect of IL-6 incorporated into liposomes.

The data shows that the percentage of pulmonary plasma cells which are antigen specific rises approximately 15-fold when IL-6 is incorporated into liposomes used for intranasal administration. The percentage of levan-specific pulmonary plasma cells were calculated by dividing the number of levan-specific pulmonary plasma cells per 10^4 isolated lung lymphocytes by the number of pulmonary plasma cells of all specificities per 10^6 isolated lung lymphocytes.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made without departing from the spirit or scope of the invention.

CLAIMS

1. A method of inducing a mucosal immune response to a polysaccharide antigen in an animal, which comprises administering to the animal an immunogenically effective amount of the antigen contained in a liposome.

2. The method of claim 1, wherein the animal has been immunocompromised.

3. The method of claim 2, wherein the administration occurs within about 48 hours of being immunocompromised, preferably within about 24 hours of being immunocompromised.

4. The method of claim 1, wherein the liposome further includes an adjuvant.

5. The method of claim 4, wherein the adjuvant is in the membrane of the liposome.

6. The method of claim 5, wherein the adjuvant is a lipoidal amine.

7. The method of claim 6, wherein the lipoidal amine is avridine.

8. The method of claim 4, wherein the adjuvant is internalized within the liposome.

9. The method of claim 8, wherein the adjuvant is a biological response modifier.

10. The method of claim 9, wherein the biological response modifier is an interleukin.

11. The method of claim 10, wherein the interleukin is IL-2 or IL-6.

12. The method of claim 8, wherein the adjuvant is complexed to the polysaccharide antigen.

13. The method of claim 1, wherein the polysaccharide antigen is a bacterial polysaccharide.

14. The method of claim 13, wherein the bacterial polysaccharide is polyvalent.

15. The method of claim 13, wherein the bacterial polysaccharide is derived from a gram-negative organism.

16. The method of claim 15, wherein the gram-negative organism is a member of the genus *Pseudomonas*.

17. The method of claim 16, wherein the organism is *Pseudomonas aeruginosa*.

18. The method of claim 13, wherein the bacterial polysaccharide is derived from a gram-positive organism.

19. The method of claim 18, wherein the gram-positive organism is a member of the genus *Streptococcus*.

20. The method of claim 19, wherein the organism is *Streptococcus pneumoniae*.

21. The method of claim 1, wherein the liposome is administered enterally.

22. The method of claim 21, wherein the enteral administration is nasally or orally.

23. The method of claim 1, wherein the animal is a human.

24. A composition useful for inducing a mucosal immune response to a polysaccharide antigen in an animal, which comprises an immunogenically effective amount of the antigen contained in a liposome, wherein at least one adjuvant is also present.

25. The composition of claim 24, wherein the adjuvant is in the membrane of the liposome.

26. The composition of claim 25, wherein the adjuvant is a lipoidal amine.

27. The composition of claim 26, wherein the lipoidal amine is avridine.

28. The composition of claim 24, wherein the adjuvant is internalized within the liposome.

29. The composition of claim 28, wherein the adjuvant is a biological response modifier.

30. The composition of claim 29, wherein the biological response modifier is an interleukin.

31. The composition of claim 30, wherein the interleukin is IL-2 or IL-6.

32. The composition of claim 28, wherein the adjuvant is complexed to the polysaccharide antigen.

33. The composition of claim 24, wherein an adjuvant is in the membrane of the liposome and an adjuvant is internalized within the liposome.

34. The composition of claim 33, wherein the adjuvant in the membrane of the liposome and the adjuvant internalized within the liposome are different.

35. The composition of claim 24, wherein the liposome is unilamellar.

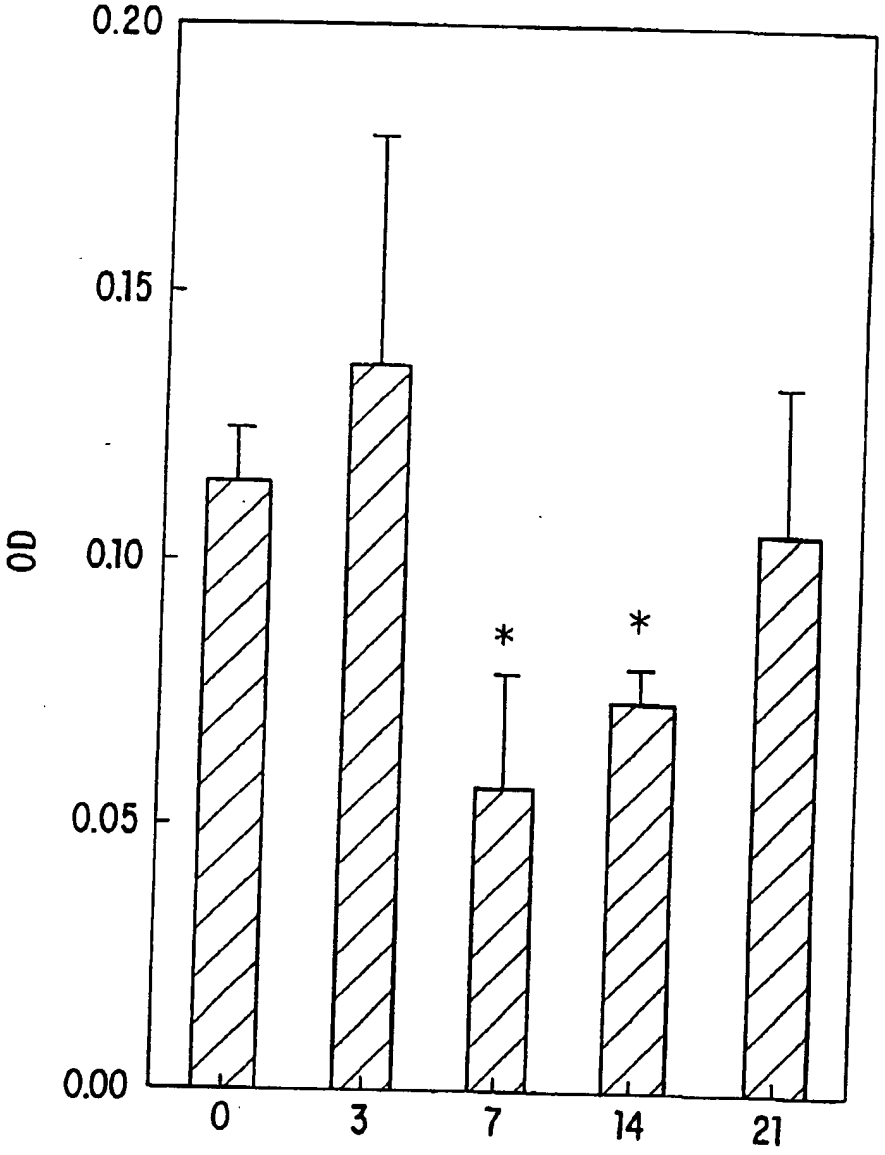
0.20
0.15
0.10
0.05
0.00
OD
*
*
0
3
7
14
21
Time of Immunization post Hemorrhage
(days)

FIG. 1

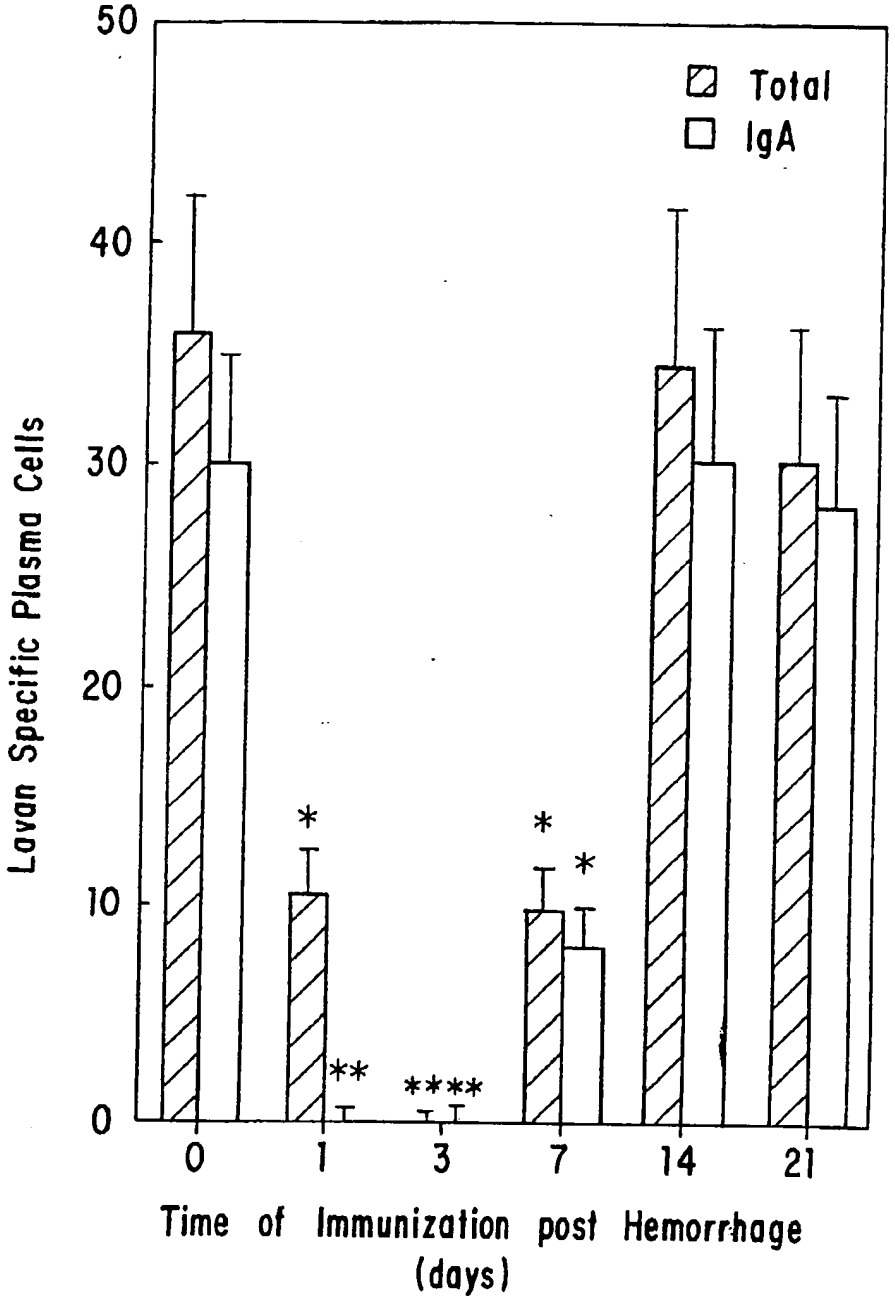
50
40
30
20
10
0
Total
IgA
Lavan Specific Plasma Cells
*
**
****
*
*
0
1
3
7
14
21
Time of Immunization post Hemorrhage
(days)

FIG. 2

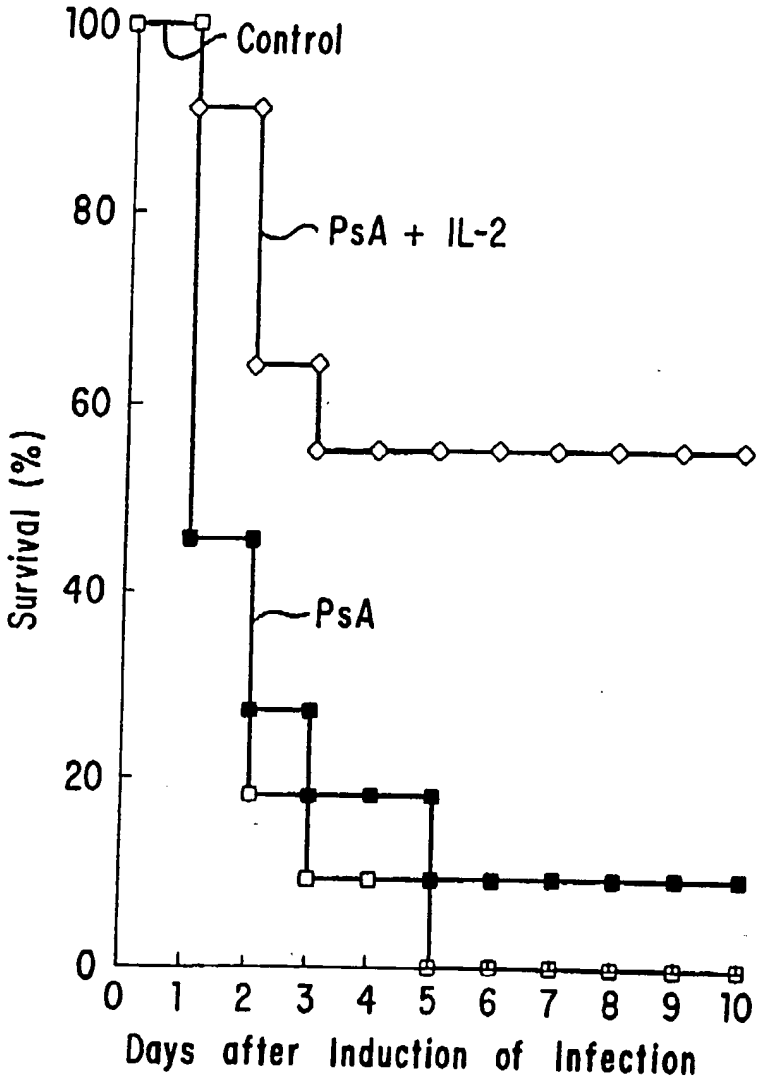
100
80
60
40
20
0
Survival (%)
Control
PsA + IL-2
PsA
0 1 2 3 4 5 6 7 8 9 10
Days after Induction of Infection

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/04055

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Immunology Volume 68, issued 1989, Davis et al, "Primary immune Response to Liposomal Tetanus Toxoid in Mice: The Effect of Mediators", pages 277-282, see page 281, left column first paragraph.	9-11, 30, 31